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SOCS3 and *IRS-1* gene expression differs between genotype 1 and genotype 2 hepatitis C virus-infected HepG2 cells

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Abstract

Background: The poor response to antiviral treatment of hepatitis C virus (HCV)-infected patients with genotype 1b has been associated with a higher prevalence of metabolic syndrome. However, the molecular link between these clinical entities is not clear. The goal of this study was to clarify the role of genotype 1b and 2 in the genetic expression of suppressor of cytokine signaling 3 (SOCS3) and insulin receptor substrate 1 (IRS-1).
Methods: We infected human hepatocellular carcinoma cell line (HepG2) cells with human HCV genotype 1b or 2 and measured the gene and protein expression of SOCS3 at various times. We also evaluated impairment in the insulin pathway by analysis of IRS-1 and phospho-AKT. For the control, we used HepG2 cell cultures treated with non-infectious serum. We also demonstrated the occurrence of HCV infection by the detection of both positive and negative strands in the cells and culture medium. To test infection of the HepG2 cells, we performed quantitative real-time polymerase chain reaction (qRT-PCR) of viral load at different time points. We analyzed the viral genotype in the pellet and supernatant.
Results: At each time point, we found positive and negative strands in the infected cells, while in the medium we found positive, but no negative strands. We also detected the presence of the correct genotype in the medium. Two weeks following infection when the viral load was higher, we tested genotype 1b and 2 infected cells. *SOCS3* gene expression was significantly higher in genotype 1b-infected cells (median 2.56; mean 2.82 ± 0.59) compared with genotype 2 (median 1.34; mean 1.46 ± 0.31) ($p = 0.04$) and control cells (median 1.09; mean 1.02 ± 0.11, $p = 0.02$). There was no difference between cells exposed to genotype 2 and control cells. Conversely, *IRS-1* was significantly lower in genotype 1b-infected cells (median 15.97; mean 15.45 ± 0.67) compared with genotype 2-infected cells (median 16.45; mean 16.44 ± 0.01, $p = 0.04$). Statistically significant differences were seen when comparing the pAKT/AKT ratio in genotype 1b-infected cells (0.19 ± 0.034) and not genotype 1b-infected (genotype 2-infected and non-infected) cells (0.253 ± 0.004, $p = 0.03$). This inverse regulation is compatible with interactions between the molecular expression of *SOCS3, IRS-1* and phospho-AKT mediated by the genotype 1b virus.
Conclusions: Up-regulation of the *SOCS3* gene might be one of the mechanisms governing non-response to therapy and expression of insulin resistance mediated via a direct mechanism at this level of genotype 1b HCV.
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Keywords: hepatitis C virus; insulin receptor substrate 1 (IRS-1); suppressor of cytokine signaling 3 (SOCS3).

Introduction

Therapy with peginterferon α and ribavirin is the standard of care for hepatitis C virus (HCV)-related chronic hepatitis (1, 2). This treatment results in a high percentage of sustained response and depends primarily on the type of infection; 90% in genotype 2 viral infection and 50% in genotype 1 viral infection (3). We previously identified a link between the response to antiviral treatment of HCV-related liver disease and a variety of genes, some of which are up-regulated and others down-regulated (4). Recently, we found that the suppressor of cytokine signaling 3 (*SOCS3*) gene, which affects the insulin pathway, was over-expressed in non-responding patients with genotype 1b HCV compared with patients with genotype 2 HCV. Also, the clinical expression of insulin resistance was significantly higher in patients with genotype 1 (5). Alterations in the insulin receptor substrate 1 (*IRS-1*) may induce insulin resistance via defects in intracellular transduction signaling (6).

SOSC3 expression has been found to be increased in liver tissue from patients with chronic genotype 1 HCV infection who did not respond to treatment (7). Huang et al. (8) confirmed this finding in cell cultures and chimpanzees. They postulated that HCV affects *SOCS3* expression by stimulating cytokines, such as TNF-α and IL-10. Subsequently, we showed that up-regulation of *SOCS3* in non-responding patients is

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related to a peculiar polymorphism of the gene, namely, –4874 A/G (9).

The goal of this study was to verify the in vitro effect of HCV on *SOCS3* overexpression in order to determine whether the level of *SOCS3* expression differs between infection with HCV genotype 1 and 2, and whether this difference might interfere with the protein concentrations of IRS-1.

Materials and methods

Viral inoculation and sample collection

Human hepatocellular carcinoma cell line (HepG2) culture and infection were performed as described previously (10–12). For infection of serum, 50 μL of patient serum was diluted in 1.5 mL of medium and added to cells plated in 35 mm dishes. Following an overnight incubation, the cells were rinsed 6–10 times with phosphate buffered saline (PBS) and 2 mL of fresh growth medium was added. The final wash was collected for HCV testing (shown as time 0 in the Figures). Cells were grown for 48 h to semi-confluence in complete medium, washed twice with fetal bovine serum (FBS)-free medium, then inoculated with serum samples from four patients infected with HCV genotype 1b, four patients with HCV genotype 2 [real-time polymerase chain reaction (RT-PCR) and antibody positive], and four healthy subjects who served as negative controls (500 μL serum and 500 μL FBS-free Dulbecco's modified Eagle medium [DMEM]/3×10^6 cells). The HCV genotypes were characterized as genotype 1b and genotype 2 as previously reported (5, 13). The viral load in sera was quantified using RT-PCR, and the average copy number was 1.9×10^5 copies/L. After 90 min, DMEM (GIBCO-Invitrogen, Milan, Italy) containing 10% FBS (Celbio, Milan, Italy) was brought to a final volume of 8 mL, including the volume of human serum used for infection as reported above. Cells were maintained overnight at 37°C in 5% CO_2. The following day, adherent cells were washed three times with culture medium to remove any remaining serum, and cell growth was continued for 4 weeks in complete medium containing 10% FBS, with a change of medium every 3 days. Cells and supernatant were collected on day 3, and 1, 2, 3 and 4 weeks after inoculation with HCV. The cellular level of viral infection was quantified at the various time points with RT-PCR amplification of sense and anti-sense strands. Genotyping analysis was also performed at these same points and genotypes were tested on the cells and culture medium.

Extraction and quantification of HCV-RNA

HCV-RNA was isolated from HepG2 cells using the guanidinium isothiocyanate method with TRIzol reagent (Invitrogen, Milan, Italy) (14), and from 200 μL of culture medium and 200 μL of serum using High Pure Viral RNA Kit (Roche Diagnostics, Milan, Italy).

Extracted RNA was amplified and quantitated using sequence-specific hybridization probes with the Reaction Mix LightCycler RNA Master HybProbe (03018954001 – Roche Diagnostics, Milan, Italy) with the LightCycler system (Roche Diagnostics, Milan, Italy). The amplification primers are located in the highly conserved 5′ untranslated (UTR) region of the HCV genome, which identifies an amplicon of 244 bp. Their sequences are: sense 5′-CTC gCA AgC ACC CTA TCA ggC AgT-3′ and antisense 5′-gCA gAA AgC gTC TAg CCA Tgg CgT-3′ (TIB MOLBIOL, Berlin, Germany).

The sequences of hybridization probes are: the donor probe 5′-gCA gCC TCC Agg ACC CCC C-3′ labeled with 5,6-carboxylfluorescein attached to 3′-O-ribose. The adjacent acceptor probe 5′-CCC ggg AgA g CC ATA gTg gTC Tg-3′ is labeled with LightCycler Red 640 attached to the 5′ terminus (both from TIB MOLBIOL, Berlin, Germany). Following hybridization to the template DNA, the two probes are in close proximity resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. The emitted fluorescence of the LightCycler Red 640 is then measured with the LightCycler Instrument. The RT-PCR reaction was performed in a final volume of 20 μL, with 8 μL of RNA. The thermal profile was 30 min at 61°C, 95°C for 30 s, followed by 45 cycles of 1 s at 95°C, 10 s at 56°C, and 1 s and 13 s at 72°C. An external standard curve to quantify the HCV genomes present in the sample was constructed using a control target 5′UTR fragment cloned from patients infected with HCV (15).

The assay targets the 5′-UTR region, and the primers are also used in the Amplicor system. The detection limit of the assay was 10 IU/mL, corresponding to ~25 copies/mL (according to the WHO, 1 IU corresponds to 2–5 genome-copies, depending on the HCV-RNA method).

PCR on HCV genomic RNA strands

Extracted RNA was analyzed using a strand-specific reverse transcriptase (RT)-nested PCR assay with Tth DNA Polymerase for positive and negative strands of HCV-RNA. In the

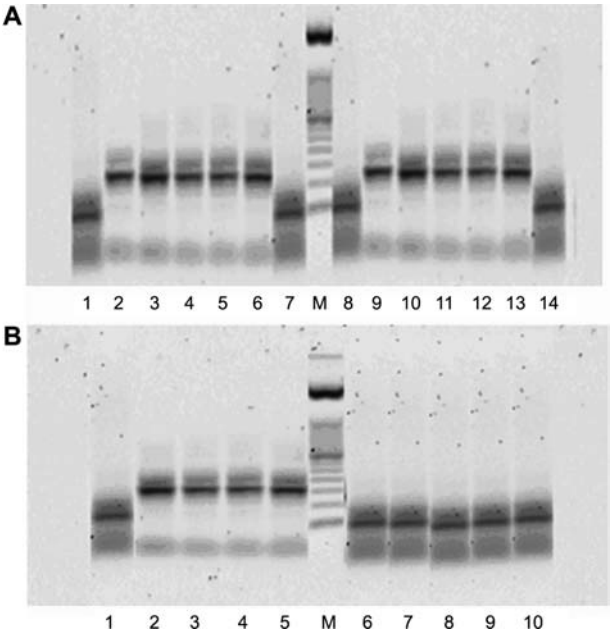

Figure 1 Nested RT-PCR amplification of viral sense and anti-sense HCV strands.
(A) HCV-RNA extracted from HepG2 inoculated with HCV-infected sera was amplified with specific primers to obtain a fragment of 154 bp in positive samples for both sense strand (from lane 2 to lane 6) and anti-sense strand (from lane 9 to lane 13). Lanes 1 and 8 represent culture cells treated with non-infectious serum and lanes 7 and 14 H_2O. (B) HCV-RNA was extracted from culture medium of HepG2 cells inoculated with HCV-infected sera at various time points during infection. Nested RT-PCR showed the presence of sense strand (from lane 2 to lane 5) in culture medium, whereas no signal was detected in the presence of anti-sense strand (from lane 7 to lane 10). Lane 1 represents culture cells treated with non-infectious serum.

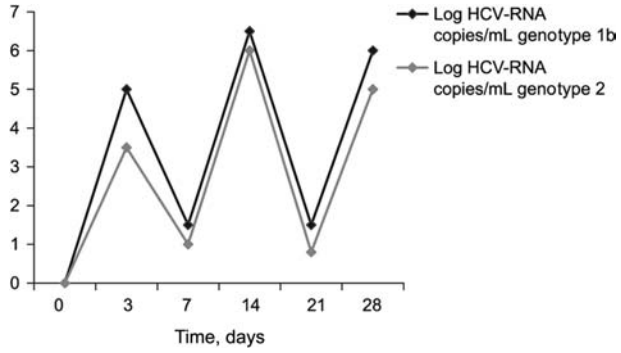

Figure 2 Viral load in HepG2 cells performed with qRT-PCR. Monitoring of infection in HepG2 cells treated with HCV infected sera of both genotype 1b and genotype 2. Viral levels were monitored periodically showing fluctuations in viral load ranging from 80 copies/mL to 6.7×10^6 copies/mL.

sense strand HCV-RNA assay, 0.5 μg of cellular RNA (corresponding to $\sim6\times10^5$ cells) in 10 μL of diethyl pyrocarbonate-treated water was heated at 70°C for 5 min and then immediately placed on ice. A 10-μL reaction mixture containing five units of Tth DNA Polymerase (Promega, Milan, Italy), 1X RT Buffer (Promega, Milan, Italy), 1 mM $MnCl_2$, in a final concentration of 0.2 mmol/L from each dNTP (Invitrogen, Milan, Italy), and 50 pmol of the reverse primer P1 was prepared for cDNA synthesis. Primer annealing was performed at 58°C for 2 min, followed by the RT reaction at 65°C for 30 min. To inactivate the RT activity of Tth, 30 μL of PCR mixture containing 8 μL of 10X buffer to chelate Mn^{2+}, 50 pmol of the forward primer P2 and 3.75 mM $MgCl_2$ was added. PCR was performed using the GeneAmp PCR-System 9600 apparatus (Applied Biosystems, Branchberg, NJ, USA). The reaction mixture was initially kept at 94°C for 1 min for enzyme activation, followed by 40 amplification cycles. Each cycle consisted of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s, and primer extension at 72°C for 30 s. This was then followed by a final extension step at 72°C for 7 min. PCR products were analyzed using agarose gel electrophoresis. The anti-sense strand HCV-RNA assay was performed using the same procedure except that the primers were used in reverse order. A 299-bp fragment was identified in positive samples. The second round of amplification was performed like the first round, except for the use of 50 pmol of the nested reverse primer P3 and 50 pmol of the forward primer P4. A 154-bp fragment was identified in positive samples. Primer sequences were: P1: 5′ ggT gCA Cgg TCT ACg AgA CCT C 3′; P2: 5′ AAC TAC TgT CTT CAC gCA gAA 3′; P3: 5′ TgC TCA Tgg TgC ACg gTC TA 3′; P4: 5′ ACT Cgg CTA gCA gTC TCg Cg 3′. All PCRs were performed in duplicate. Amplified products were analyzed using electrophoresis with a 2% agarose gel and stained with Vistra Green nucleic acid. The same gel was scanned with STORM 860 to 450 nm (Molecular Dynamics, GE Healthcare Europe GmbH, Milan, Italy) (16, 17).

HCV genotyping of infected cells

RNA extracted from HepG2 cells and culture medium was used for genotyping. Using reverse primer P1 in the RT step, cDNA copies were generated from viral RNA. The cDNA was amplified using PCR with outer primers in the first round and inner primers in the second round contained in the HCV Genotype Amplification kit (LIPA – SIEMENS, Bayswater VIC, Australia). DNA products were amplified and biotinylated in the Versant HCV genotype assay (LIPA – SIEMENS, Australia) (18).

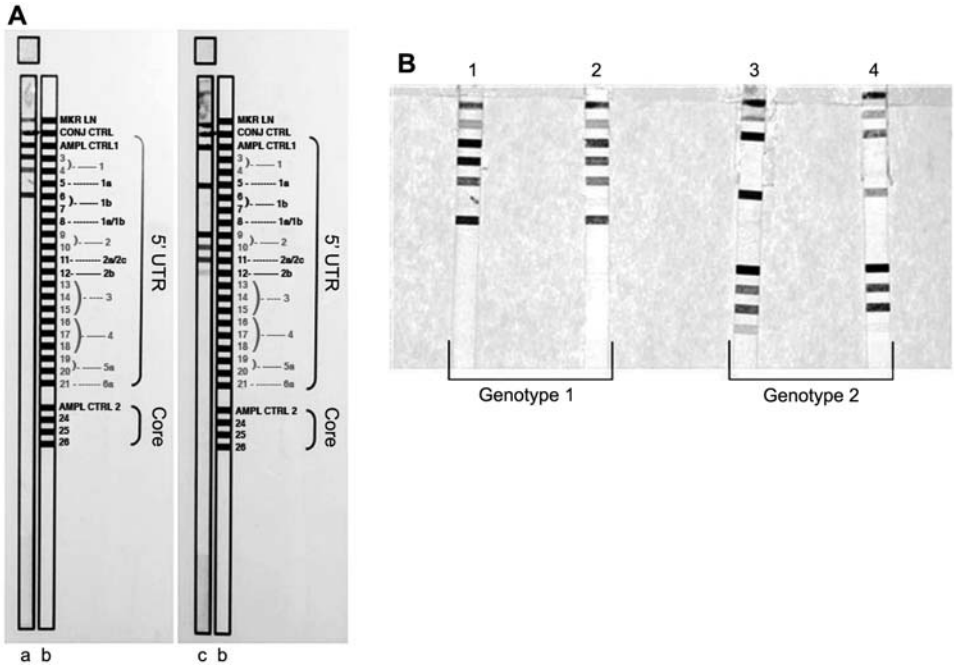

Figure 3 Genotyping of HepG2 cells and culture medium with Inno Lipa 2.0 Kit.
The Versant HCV genotype 2.0 Assay (LiPA) uses a biotinylated DNA PCR product specific to the 5′UTR and core region of the HCV genome. (A) a: the strip identifying probes that hybridize the PCR product of serum samples with genotype 1b HCV; b: the reading card to report data; c: the strip identifying probes that hybridize the PCR product of serum samples with genotype 2 HCV. (B) Presence of genotype in the cells and culture medium. Strips 1–3 represent respectively genotype 1b and 2 detected in the cells; strips 2–4 represent respectively genotype 1b and 2 detected in culture medium. Genotyping was done at every time point and for every cell culture.

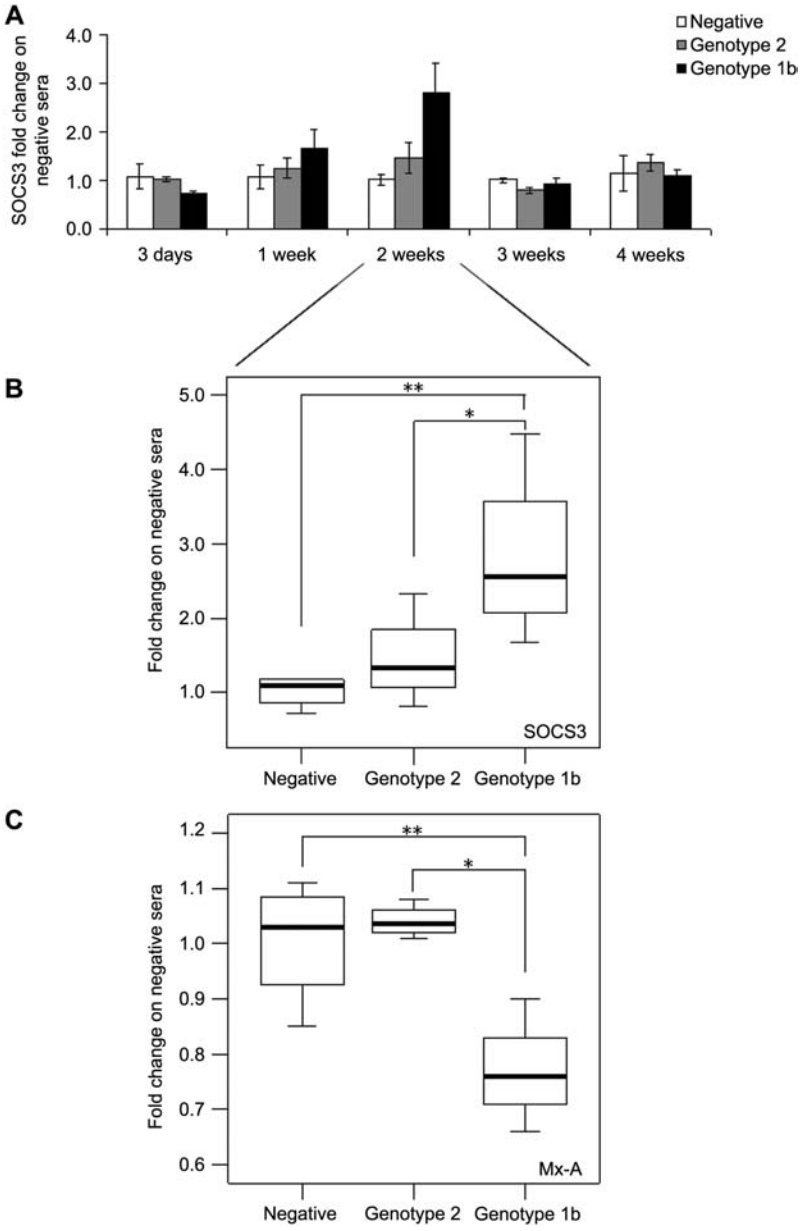

Figure 4 *SOCS3* gene expression in genotype 1b, genotype 2 and control HEPG2 cells.
(A) *SOCS3* gene expression profile at day 3, and 1, 2, 3 and 4 weeks after inoculation with HCV-infected sera. (B) After 2 weeks of HCV infection, *SOCS3* gene expression was significantly up-regulated in HepG2 cells infected with HCV genotype 1b vs. cells with genotype 2 (Mann-Whitney test; *p = 0.04) and non-infected cells (Mann-Whitney test; **p = 0.02). (C) *Mx-A* gene expression 2 weeks after HCV infection. Data are presented as medians and means ± SEM.

RNA isolation, RT-PCR and quantitative real-time (qRT)-PCR analysis of SOCS3, Mx-A and IRS-1

Total cellular RNA was extracted from the cultured cells using the guanidinium isothiocyanate method with the TRIzol reagent (Invitrogen, Milan, Italy) (19). All RT reactions were performed using the iScript™ cDNA synthesis kit (BioRad, Milan, Italy), according to the manufacturer's instructions. qRT-PCR was performed using the SYBR Green PCR Master Mix with the Applied Biosystem Model 7900HT Sequence Detection System. The primers were designed by the Primer Express 2.1 program (Applied Biosystems, Branchberg, NJ, USA). Primer sequences are as follows: *SOCS3,* forward 5′-CTTTCTGATCCGCGACAGCT-3′, reverse 5′-TGGTCCCAGACTGGGTCTTG-3′; *Mx-A,* forward 5′-GGAGGAGATCTTTCAGCACCTG-3′, reverse 5′-TGGATGATCAAAGGGATGTGG-3′; *IRS-1,* forward 5′-GCCAGAGGACCGTCAGTAGCT-3′, reverse 5′-AGGATTTGCTGAGGTCATTTAGGT-3′. All PCRs were performed in duplicate. β-Actin (forward 5′-CGTGCTGCTGACCGAGG-3′, reverse 5′-GAAGGTCTCAAACATGATCTGGGT-3′) was used as internal control.

Western blotting

Harvested cells were washed three times with ice-cold PBS and homogenized with ice-cold buffer for extracting the cytosolic fraction at 4°C [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol and a complete protease inhibitor cocktail (Roche Diagnostics, Milan, Italy)]. Protein content was measured with the Bradford assay (BioRad, Milan, Italy). Using the method of Laemmli (20), 30 μg of cytosolic lysate was loaded onto two different

Table 1 SOCS3 gene expression analysis in all experimental points.

Collection points	*SOCS3* mRNA levels [mean (SE); median]			p-Value[a]	p-Value[b]
	Negative	Genotype 2	Genotype 1b		
3 days	1.08 (0.27); 1.26	1.02 (0.09); 1.08	0.73 (0.06); 0.73	N.S.	N.S.
1 week	1.08 (0.24); 0.96	1.25 (0.19); 1.11	1.66 (0.40); 2.03	N.S.	N.S.
2 weeks	1.02 (0.11); 1.09	1.46 (0.31); 1.34	2.82 (0.59); 2.56	0.04	0.02
3 weeks	1.00 (0.05); 0.98	0.80 (0.07); 0.82	0.93 (0.12); 0.99	N.S.	N.S.
4 weeks	1.14 (0.37); 0.89	1.36 (0.17); 1.39	1.10 (0.12); 1.07	N.S.	N.S.

[a]Comparison between cell lines infected by HCV genotype 2 vs. genotype 1b; [b]comparison between cell lines infected by HCV genotype 1b vs. those treated with negative sera Mann-Whitney test. *SOCS3,* suppression of cytokine signaling 3; SE, standard error; N.S., not significant.

polyacrylamide gels: 12% for SOCS3 protein, 6% for IRS-1 protein and 10% for AKT and phospho-AKT (Ser473) proteins. Western blotting was performed as described elsewhere (21) with a specific mouse anti-SOCS3 antibody (1:500) (BioLegend, San Diego, CA, USA), a specific rabbit anti-IRS-1 (1:500), a specific rabbit anti-AKT (1:1000) and phospho-AKT (Ser473) (1:500) (Cell Signaling Technology, Danvers, MA, USA). Goat anti-β-actin antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the control for equal loading. Semiquantitative analysis of protein expression was performed. The bands were quantified by densitometry to obtain an integral optical density value, which was then normalized with respect to the β-actin value. We used the GS-800 Calibrated Densitometer and the Quantity One 4.6.3 program (BioRad, Milan, Italy) for densitometric analyses.

Gene and protein expression analysis

For qRT-PCR data, the mean fold change $=2^{-(\text{average } \Delta\Delta Ct)}$ was calculated using the mean difference in the ΔCt between the genes and the internal control. The ΔCt was calculated using the differences in the mean Ct between selected genes and the internal control. Ct indicates the cycle threshold, the fractional cycle number where the fluorescent signal reaches the detection threshold (22). The significance of difference in protein and gene expression was determined using the Mann-Whitney or Student t-tests. A $p<0.05$ was used for statistical significance.

Results

HCV replicates in HepG2 cells infected in vitro

HCV infection was monitored using nested RT-PCR amplification of viral sense and anti-sense HCV strands. Figure 1A shows HCV infection in HepG2 cells; both positive and negative HCV-RNA strands were found in cell pellets. To verify infection, we performed the same experiment with the culture medium. As shown in Figure 1B, positive HCV-RNA strand appeared, but, as expected, the negative strand did not (Figure 1B). Similar results were obtained with HCV genotype 1- and 2-infected cells at each reported time (see ''Materials and methods''). Following treatment with HCV positive sera and negative sera, we monitored infection in the cultures with qRT-PCR for 28 days. HCV-RNA shows a cyclical pattern, with peaks at 3, 14, 28 days following infection (Figure 2). Fluctuations in HCV levels were observed in both cultures infected with genotype 1b and genotype 2 sera. HCV levels in the cultures reached high concentrations, ranging from 10^5 to 10^7 copies/mL during the 4 weeks culture period. Also, we used the genotyping hybridization method as the control for the experiment and infection. Figure 3 shows data documenting the genotype 1b and 2 infection of sera used for HepG2 cells incubation (panel A), and for supernatant and cell cultures (panel B).

SOCS3 gene expression and protein levels in HepG2 cells infected in vitro

We measured *SOCS3* gene expression by qRT-PCR in HepG2 cells at day 3, and 1, 2, 3 and 4 weeks following inoculation with HCV (Figure 4A). *SOCS3* gene expression did not differ significantly between cell lines infected with HCV genotype 1b and those infected by HCV genotype 2, nor did they differ between HCV genotype 1b-infected cells and cells treated with

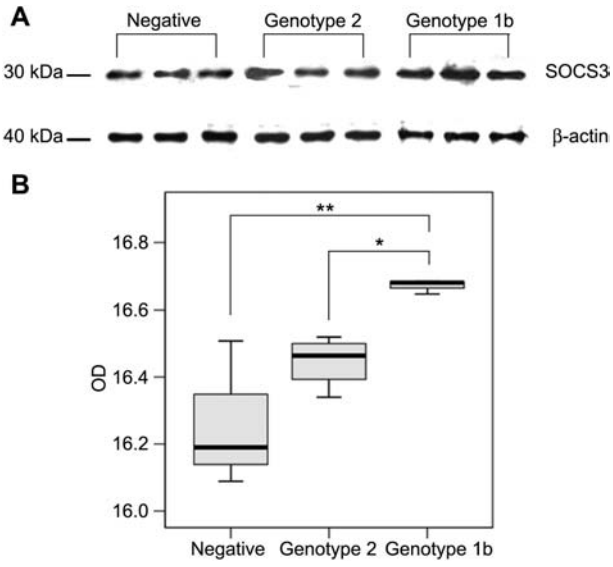

Figure 5 Levels of the SOCS3 protein in HepG2 2 weeks after HCV infection.
(A) Western blot of SOCS3 and β-actin in HepG2 cells inoculated with serum from 3 HCV genotype 1b- and 3 HCV genotype 2-infected patients; 3 healthy, HCV-negative subjects served as negative control. (B) The protein level was significantly higher in cells infected with HCV genotype 1b vs. cells infected with HCV genotype 2 (Mann-Whitney test; *$p=0.04$) and non-infected cells (Mann-Whitney test; **$p=0.03$). Data are presented as median and mean ± SEM. OD, optical density.

healthy control sera, at day 3, and 1, 3 and 4 weeks following inoculation with HCV (Table 1). Two weeks following HCV infection, *SOCS3* mRNA levels were significantly higher in cells infected with HCV genotype 1b (median 2.56; mean 2.82±0.59) vs. cells infected with genotype 2 (median 1.34; mean 1.46± 0.31, p=0.04) and vs. cells that were not infected (median 1.09; mean 1.02±0.11, p=0.02) (Figure 4A and B). Genotype 2 cells did not differ from control cells. There was an inverse relationship between *SOCS3* gene expression and the expression of *Mx-A* (Figure 4C), which is consistent with previous data (9). In particular, *Mx-A* was down-regulated significantly in HepG2 cells infected with HCV genotype 1b (median 0.76; mean 0.77±0.05) compared with cells infected with genotype 2 (median 1.04; mean 1.04±0.01, p=0.02) and cells that were not infected (median 1.03; mean 1.01±0.06, p=0.04). Western blot analysis confirmed that SOCS3 protein levels were significantly higher in cells infected with genotype 1b (median 16.68; mean 16.67±0.01) with respect to genotype 2 (median 16.48; mean 16.48±0.02, p=0.04) and control cells (median 16.19; mean 16.26±0.13, p=0.03) (Figure 5A and B).

IRS-1 gene expression and protein levels in HepG2 cells infected in vitro

We analyzed *IRS-1* gene expression and IRS-1 protein levels in genotype 1b- and 2-infected cells at 3 days and 2 weeks following infection with HCV. *SOCS3* expression peaked 2 weeks following infection with genotype 1-infected HepG2 cells (Figure 1). *IRS-1* expression, measured with qRT-PCR, did not differ between genotype 2- and genotype 1b-infected cells (Figure 6A). Conversely, when measured with Western blotting, the IRS-1 protein level was significantly lower in genotype 1b-infected cells (median 15.45; mean 15.47±0.03) than in cells infected with HCV genotype 2 (median 16.60; mean 16.59±0.01, p=0.02) (Figure 6B and C). Of note, there was no difference in protein levels between HepG2 cells infected by HCV genotype 2 and cells that were not infected (p=0.15).

AKT protein levels in HepG2 cells infected in vitro

We analyzed the protein level of AKT and its phosphorylation state (pAKT) by Western blotting (Figure 7A) using the same samples that had been analyzed

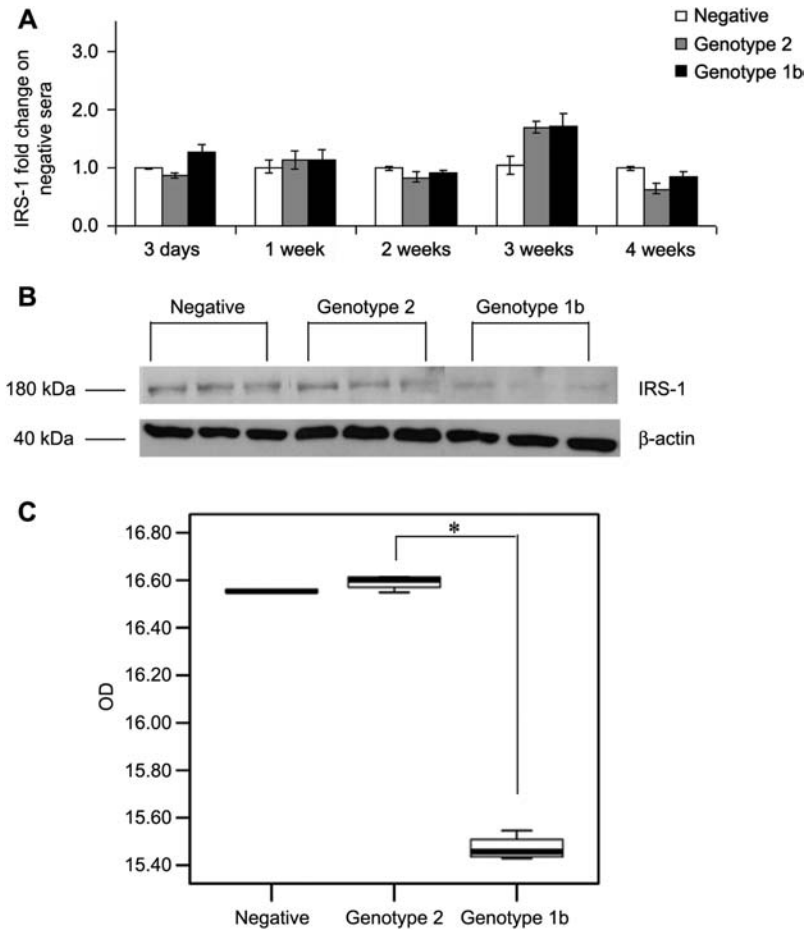

Figure 6 Insulin receptor substrate 1 (*IRS-1*) gene expression and protein level in HepG2 infected cells.
(A) *IRS-1* gene expression 3 days and 2 weeks after HCV infection. (B) Levels of the IRS-1 protein in HepG2 2 weeks after HCV infection. (C) The IRS-1 protein level was significantly lower in cells infected with HCV genotype 1b vs. cells infected with HCV genotype 2 (Mann-Whitney test; *p=0.02) and vs. non-infected cells (Mann-Whitney test; p=0.02). The y-axis shows the optical density (OD) of the IRS-1 expression. Data are presented as median and mean±SEM.

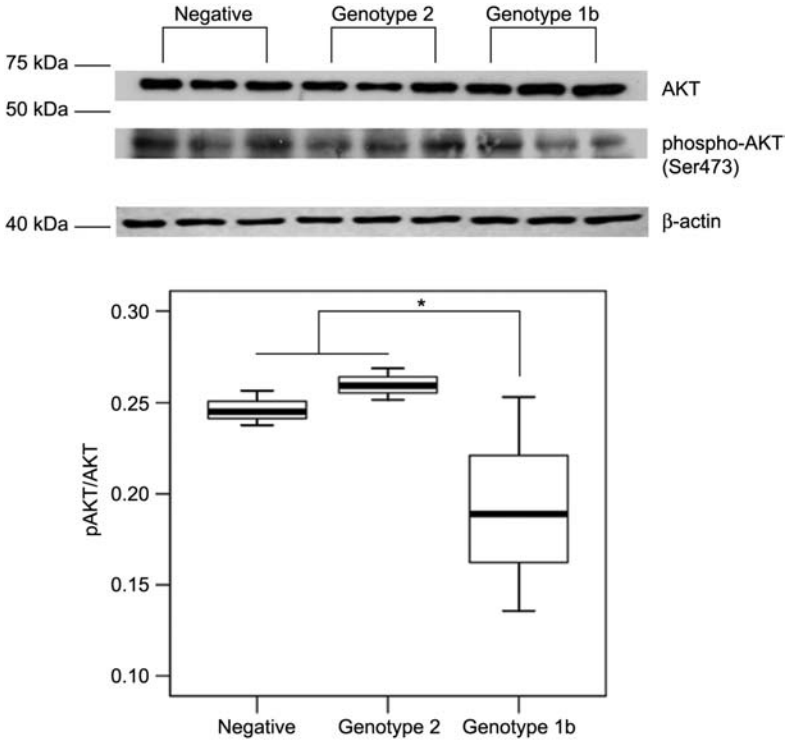

Figure 7 AKT and phospho-AKT (Ser473) protein levels in HepG2 infected cells.
(A) Levels of the AKT and phospho-AKT proteins in HepG2 2 weeks after HCV infection. (B) The pAKT/AKT ratio was significantly lower in genotype 1b-infected cells vs. cells not infected with genotype 1b (genotype 2-infected and not infected) cells (Student t-test, *$p = 0.03$). The y-axis shows the optical density (OD) of the phospho-AKT expression compared to AKT protein. Data are presented as median and mean $\pm$ SEM.

for *IRS1* and *SOCS3* expression. We observed a reduction of pAKT/AKT ratio in genotype 1b-infected cells when compared to genotype 2-infected cells (25.9%) and cells that were not infected (21.9%). A statistically significant difference was observed when comparing the pAKT/AKT ratio in genotype 1b-infected cells (0.19 ± 0.034) and cells not infected with genotype 1b (genotype 2-infected and not infected) cells (0.253 ± 0.004, $p = 0.03$) (Figure 7B).

Discussion

To evaluate if the HCV genotype affects the molecular expression of SOCS3, we reproduced genotype 1 and 2 virus infection in HepG2 cells. We used HepG2 cells rather than a lymphoblastoid cell line because their biosynthetic pathway is similar to that of hepatocytes (22), and because overexpression of SOCS1 and SOCS3 in human hepatoma HepG2 cells suppresses IFN-α-induced STAT activation and gene expression of the antiviral proteins 2′,5′ OAS (2′-5′-oligoadenylate synthetase and MxA (21). In addition, lymphoblastoid lines immortalized by Epstein-Barr virus interferes with HCV infection. Finally, propagation of HCV in HepG2 cells has been well validated (10, 23). We inoculated HepG2 cells with either genotype 1 or genotype 2 according to Lázaro et al. who reported that serum infected cells still showed high virus titers up to 2 months in both the cell pellet and the culture medium (12). In our system, cells showed fluctuating virus levels up to 30 days and greater (data not shown), in a cyclic pattern with peaks at 4, 15 and 24 days. This may reflect the effect of host responses to the virus as reported by others (12, 24, 25). Moreover, the analysis of genotypes by reproducing the genotype specific infection in both cell culture and culture medium also confirms that our system is able to support viral replication.

After 2 weeks of viral exposure, SOCS3 expression was significantly higher in cells infected with genotype 1b vs. cells infected with genotype 2, and also vs. non-infected cells. There was no difference between genotype 2-infected and non-infected cells. Of interest was the ''mirror effect'' during the peak of viremia, SOCS3, IRS-1 and AKT in genotype 1-infected cells. Namely, the HCV-RNA peak was associated with a significant increase in SOCS3 and a significant decrease in the intracellular levels of IRS-1 and pAKT. This observation is in agreement with the finding that SOCS3-related IRS-1 degradation occurs via the ubiquitin system (22, 23). Moreover, the reduction in AKT phosphorilation confirms the altered intracellular insulin metabolic pathway, and supports the idea that genotype 1b HCV might somehow interfere at this level. The inverse regulation between SOCS3 and IRS-1 suggests that genotype 1b HCV affects the expression of SOCS3 and IRS-1. These results are in agreement with our previous report of enhanced SOCS3 in non-responding patients with genotype 1 HCV infection

(5), and supports the concept that virus-related SOCS3 gene up-regulation underlies the non-response to therapy and the clinical expression of insulin resistance in genotype 1-infected patients.

Pazienza et al. (23) reported significantly greater insulin resistance in genotype 1 patients, and found that the mechanism underlying IRS-1 down-regulation was not related to SOCS3. Our data do not support this observation, and instead support the finding that the HCV down-regulates IRS-1 and 2 by up-regulating SOCS3 (26). Moreover, our finding supports the concept that the IFN-α antagonistic activity of HCV core protein involves SOCS3 induction (27). This is consistent with the association between lack of response to antiviral therapy and obesity and hepatic expression of SOCS3 in genotype 1 patients (6). However, insulin resistance is a predictor of sustained viral response in patients that respond to treatment (genotype 2 and 3) (28). The apparent discrepancy between this observation and our data could be that non-responding patients infected with ''easy'' genotypes might have a SOCS3 polymorphism that results in higher levels of SOCS3. This fact, together with jak/stat suppression, might interfere with the insulin intracellular pathway. Further studies are needed to address this hypothesis.

In conclusion, we demonstrate that genotype 1b HCV plays a crucial direct role in up-regulation of the *SOCS3* gene, conditioning the response to antiviral therapy and expression of insulin resistance. This observation challenges the concept that the response to therapy is governed by insulin resistance. Further studies on the biological mechanism underlying the interaction between HCV and SOCS3 might lead to new combinations of therapeutic strategies that are effective in patients who do not respond to traditional therapy.

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